1759-Pos Board B489
Conformational and Functional Effects of Pathogenic Mutations at the I-T Interface of Cardiac Troponin I
Shirin Akhter, J.-P. Jin.
Physiology, Wayne State University, Detroit, MI, USA.
Transgenic mouse hearts expressing K118C (K117C in human) mutation in cardiac troponin I (TnI) at the I-T interface exhibited decreased diastolic function and blunted beta-adrenergic response (Wei et al., JBC 285:27806, 2010). An adjacent mutation, A116G, in human cardiac TnI was found in cardiomyopathy (Millat et al., Eur J Med Genet 54:e570, 2011), further indicating the pivotal role of the I-T interface of TnI in troponin regulation of muscle contraction and cardiac function. N-terminal truncation and PKA phosphorylation of cardiac TnI result in similar conformational change in the I-T helix region and similar effects on the interaction with troponin C (Akhter et al., AJP Heart Circ Physiol. 302:H923, 2012). To understand the function of the I-T interface of TnI, we constructed K118C and A117G mutations in intact as well as in N-terminal truncated mouse cardiac TnI. Using recombinant proteins purified from bacterial culture, conformational analysis and protein binding studies were carried out. K118C and A117G mutations both significantly altered the mobility of the TnI protein in SDS-PAGE, indicating major conformational modulations. Despite their adjacent locations, A117G mutation results in faster gel mobility of cardiac TnI whereas K118C mutation decreases the mobility of cardiac TnI in SDS-gel. The changes in protein conformation are accompanied with functional alterations. K118C mutant decreased the binding affinity for troponin C in a Ca^{2+}-dependent manner, while A117G had a similar but less profound effect. Restrictive truncation to remove the cardiac specific N-terminal extension minimized the effect of both mutations, suggesting a posttranslational mechanism to compensate for the conformational and functional abnormality of the I-T interface mutations.

1760-Pos Board B490
Computational Prediction and Experimental Verification of Differential Calcium Affinity in Thin Filament Mutants Known to Cause Hypertrophic Cardiomyopathy
Edward P. Manning[1], **Sarah J. Lehman**[2], Steven D. Schwartz[3], Jil C. Tardiff[4].
[1]Physiology and Biophysics, Einstein College of Medicine, Bronx, NY, USA, [2]Physiological Sciences, University of Arizona, Tucson, AZ, USA, [3]Chemistry and Biochemistry, University of Arizona, Tucson, AZ, USA, [4]Cellular and Molecular Medicine, University of Arizona, Tucson, AZ, USA.
Alterations in the calcium affinity of cardiac troponin C (cTnC) with eventual effects on cardiac physiology have been known to result from thin filament mutations in cardiac Troponin T (cTnT) that cause hypertrophic cardiomyopathy (HCM). In this work we report on first principles computational predictions of calcium binding affinity as predicted by coordinating oxygen distance. These calculations are in both a phosphorylated and non-phosphorylated state at serines in positions 23 and 24 in the inhibitory protein of the troponin complex (cTnI,) and so represent the lowest level myofilament effects of adrenergic signaling. The predictions are made using an all atom molecular model of the troponin complex and tropomyosin developed in our two research groups. In order to test the validity of these predictions, IAANS measurements of calcium affinity in fully recombinant thin filaments were performed using a phosphomemetic cTnI (cTnI-DD). Preliminary steady-state results with the wild-type (phosphomimetic) cTnI replicated the predicted decrease in Ca^{2+} sensitivity. In the same fashion, atomistic calculations showed longer distances between the coordinating oxygens in the phosphorylated state as compared to the unphosphorylated state. These longer distances correspond to weaker Ca^{2+} binding and decreased sensitivity. The effects of substitutions in cTnT are in progress and will be presented along with the computational work. This work represents the beginning of ab initio prediction of disease effects at the molecular level via the use of validated computer simulation.

1761-Pos Board B491
Ca^{2+}-Induced Structural Changes in Tn: A Multi-Site FRET Study Combining TCSPC with Single Filament Imaging
Maria E. Moutsoglou, Gi-Ho Kim, Christopher Solis-Ocampo, Steven C. Wu, John M. Robinson.
Chemistry and Biochemistry, South Dakota State University, Brookings, SD, USA.
Troponin is the allosteric sensor for calcium in cardiac muscle. During activation, calcium binds to troponin (Tn), causing a conformational change leading to myofilament activation. We seek to understand the energetics and design principle of the regulated actin (TnTmA7) switch and the basis for cooperative activation in cardiac muscle contraction. To study Ca^{2+}-induced structural changes in Tn, we have engineered single Cys-mutants within Tn that enable the measurement of 36 unique inter-probe distances by time-resolved FRET (trFRET). Regulated actin (rAc) was reconstituted from dye-labeled Tn, tropomyosin (Tm) and f-actin (A7), immobilized on glass coverslips. Epifluorescence images revealed actin filaments with no TnTm bound, actin filaments fully decorated with TnTm, bundles of rAc, and random points of fluorescence from Tn and Tm not bound to actin. Video microscopy demonstrated Brownian movement in non-immobilized segments of rAc. To obtain reliable trFRET data from Tn within non-bundled rAc filaments, we first imaged partially immobilized rAc. Isolated filaments were identified, and TCSPC was performed on a single point within the filament for 1 min, yielding 20,000 photons from approximately 50 Tn molecules within the 1 μm3 confocal volume. The inter-dye distance was obtained under Mg^{2+}- and Ca^{2+}-saturating conditions in 36 FRET constructs.

1762-Pos Board B492
Partial Activation of the Cardiac Myofilament by Ca^{2+}
Mathivanan Chinnaraj.
Waksman Institute of Microbiology, Rutgers, The State University of New Jersey, Piscataway, NJ, USA.
The cardiac myofilament is a protein assembly that enable the heart to undergo alternating periods of contraction and relaxation, the driving force regulated by Ca^{2+}. Troponin, a three-member protein assembly within the myofilament, acts as a Ca-sensitive switch. In this work, we used single molecule FRET technique to monitor whether the Troponin complex functions as a Ca^{2+} -sensitive regulatory switch . The results show a population of unactivated troponin under saturating Ca^{2+} conditions. We propose that the population of unactivated troponin comprises a form of cardiac reserve that is regulated by signaling pathways that target the myofilament.

1763-Pos Board B493
Modulation of the Interaction between Troponin I N-Terminal Peptide and Troponin C by Phosphorylation Studied by Molecular Dynamics
Ian Gould, Andrew E. Messer, Maria Papadaki, **Steven B. Marston**.
Imperial College London, London, United Kingdom.
The Ca^{2+}-sensitivity of cardiac thin filaments is modulated by interaction between the N terminal peptide of troponin I and the N terminal lobe of troponin C that enhances Ca^{2+} sensitivity. The interaction is abolished by PKA phosphorylation of Ser 22 and 23. We have applied molecular dynamics simulations to the Takeda et al. structure of the core domain of human cardiac troponin in explicit water on an expanded model of the full crystallographic structure (385 amino acids). The crystal structure is deficient in the first 31 residues of TnI which we have added in as a linear chain above the TnC N-terminal lobe according to the model of Howarth et al., (J Mol Biol 373 , 709). All simulations have been performed for a quarter of a microsecond with the AMBER GPU MD package in an isobaric-isothermal, NPT, ensemble.
In the Ca^{2+}-bound unphosphorylated state there is a persistent interaction between Arg 20 through Ser 24 of TnI and the Ca^{2+}-binding loops of Tn-C that could modulate Ca^{2+}-binding. The extreme N-terminal 1 to 16 Amino acids of TnI are very mobile.
Phosphorylation leads to restructuring after 50 nsecs with reduction of strong TnI-TnC contacts and a re-orientation of the TnC N-terminal lobe relative to the rest of troponin. Ca^{2+} becomes more exposed to solvent. Between 100 and 150ns the Ca^{2+} dissociates and reassociates. Between 150 and 200ns the first 20 amino acids of TnI form a beta sheet structure that interacts with alpha helices Lys39 - Leu 48 and Pro 54 and Ile 61 of TnC. The adjacent TnI 43-59 helix that normally binds in a cleft of the C-terminal lobe of troponin C is also perturbed, indicating long range effects of phosphorylation.

1764-Pos Board B494
Cardiac Troponin I A164H and pH-Dependent Inotropy
Anthony D. Vetter[1], Brian Thompson[1], Joseph M. Muretta[2], David D. Thomas[2], Joseph M. Metzger[1].
[1]Integrative Biology and Physiology, University of Minnesota, Minneapolis, MN, USA, [2]Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, Minneapolis, MN, USA.
Ischemic heart disease is the most common cause of mortality in the world. To develop novel therapeutic strategies and gain mechanistic insight we are using time-resolved fluorescence measurements to detect structural changes within the cardiac troponin complex that occur during acute ischemic insult. During myocardial ischemia, penultimate to cell death, there is a shift from oxidative to glycolytic metabolism resulting in an acidification that severely impairs sarcomere function. In cardiac myocytes, this decrease in pH uncouples calcium homeostasis from force-generation. We and others have shown

that the neonatal cardiac isoform of TnI, ssTnI, confers pH-insensitivity in this regard compared to the adult cTnI isoform. However ssTnI confers deleterious effects of impaired relaxation in the adult myocyte. Alignment and functional studies have demonstrated that this pH-insensitivity is derived from ssTnI residue H132. Introduction of a histidine at the cognate position in cTnI (A164H) mitigates the pH-sensitivity of the calcium-force relationship in cardiac myocytes while retaining relaxation enhancement via the N-term domain relative to ssTnI. We are establishing a time-resolved fluorescence methodology for detecting alterations in the calcium sensitivity of the thin filament during ischemia. We have engineered a single cysteine mutation for labeling with environmentally sensitive fluorophores designed to detect Ca^{2+} and pH-sensitive structural changes in cTnI and cTnC. We will discuss progress using this approach to interrogate troponin function in ischemia mimetic conditions.

1765-Pos Board B495

Molecular Mechanism of Cardiomyopathy-Causing Mutations in Alpha-Tropomyosin

Tejas M. Gupte[1], Farah Haque[1,2], Binnu Gangadharan[1,3], Margaret Sunitha[2], Suman Nag[4], Sowdhamini R[2], VijayRaghavan K[2], James A. Spudich[1,4], John A. Mercer[1,5].
[1]Cardiomyopathy group, inStem, Bangalore, India, [2]NCBS-TIFR, Bangalore, India, [3]MAHE, Manipal, India, [4]Biochemistry, Stanford University, Palo Alto, CA, USA, [5]McLaughlin research Institute, Great Falls, MT, USA.

Primary cardiomyopathy is one of the most common cardiac disorders, affecting more than 1 in 500 individuals. Primary cardiomyopathies are most frequently caused by inherited single amino acid substitutions, in a single allele encoding one of the cardiac sarcomeric proteins.

Alpha-tropomyosin is a key cardiac sarcomeric protein, which interacts structurally and functionally with all other components of the sarcomeric contractile apparatus and thereby regulates cardiac muscle contraction in response to Ca^{2+}. There are more than fifteen substitutions identified throughout the length of alpha-tropomyosin which can result in cardiomyopathy. However, the fundamental biochemical and biophysical mechanism(s) by which these single amino acid substitutions affect sarcomeric function and cause cardiomyopathy is (are) unclear. Also, there is no clear relation between the location of these substitutions in alpha-tropomyosin and the nature of the resulting cardiomyopathy. Working with a collection of less-characterised, cardiomyopathy-associated mutations in human cardiac alpha-tropomyosin, we find that even if two mutations are associated with the same cardiomyopathy, the molecular dysfunction caused by the two mutations could be different. Previously it has been characterized that most HCM mutations in alpha-tropomyosin show weaker binding to actin. However, we find that the HCM-associated alpha-tropomyosin L185R mutant binds to F-actin with a greater affinity and co-operativity. In a co-sedimentation assay to measure the binding of alpha-tropomyosin and F-actin, the Kd decreases from 200 ± 20 nM (wild-type) to 100 ± 30 nM (L185R), and the Hill's coefficient increases. This mutation, and some others, have been characterized in further detail, within the frame-work of the three-state model of the regulated thin filament, to provide novel insights into the mechanisms underlying cardiomyopathies.

1766-Pos Board B496

Skeletal Muscle Myopathy Mutations in Tropomyosin Gene TPM3 affect Thin Filament Transitions between the Inactive and Active States

Mohammed El-Mezgueldi[1], Saeed Asiri[1], Widad Albishri[2], Kristen Nowak[3].
[1]Biochemistry, University of Leicester, Leicester, United Kingdom, [2]Chemistry, King Abdul Azziz University, Jeddah, Saudi Arabia, [3]Biochemistry, The University of Western Australia, Nedlands, Australia.

It has recently been discovered that nemaline myopathy and cap myopathy can be caused by mutations in β-tropomyosin (*TPM2* gene) and that a third skeletal muscle myopathy, congenital fibre-type disproportion, is predominantly due to mutations in slow α-tropomyosin (*TPM3* gene). We have expressed tropomyosin TPM3 mutations of residues R90, R167 and R244 in a baculovirus-Sf9 insect cells system and studied reconstituted actin-tropomyiosin mutant filaments biochemical properties. We analysed the effects of these TPM3 mutations on the biochemical properties of tropomyosin namely secondary structure, actin binding and the equilibrium constants of transitions between the 'open', 'closed' and 'blocked' states of thin filament and the cooperativity of these transitions. We also assessed their functional effects on the actomyosin ATPase. We found that TPM3 mutations did not affect secondary structure of tropomyosin and its interaction with actin. In contrast these mutations affected troponin-tropomyosin inhibition and activation of actomyosin ATPase. TPM3 mutations also affected the Blocked-closed transitions in the cooperative allosteric mechanism of regulation of the actomyosin complex. These findings are in agreement with recent structural studies of tropomyosin bound to actin in the closed and blocked conformations. We propose that destabilisation of the actin-tropomyosin interface by charge change in the blocked state may represent the underlying biochemical defect for the onset of skeletal muscle myopathies linked to these mutations.

1767-Pos Board B497

Direct Visualization of Cooperative Binding of Troponin-Tropomyosin to F-Actin

Christopher Solis-Ocampo, Maria Moutsoglou, Gi-Ho Kim, John M. Robinson.
Chemistry & Biochemistry, South Dakota State University, Brookings, SD, USA.

In cardiac muscle, troponin (Tn) and tropomyosin (Tm) on f-actin (Ac) provide Ca^{2+}-dependent regulation of force development by myosin motors. Ca^{2+} activation is a highly cooperative process that is thought to be due to cooperative structural interactions among Tn, Tn, and Ac. Previous measurements have quantified cooperative binding associations through indirect methods coupled with modeling. We sought to characterize cooperative interactions within regulated actin by directly observing individual actin filaments. Tn, Tm, and Ac were labeled, respectively, with AF 546 (green), ATTO 655 (red) and phalloidin AF488 (blue dyes). Regulated actin was reconstituted at 1:1:1 stoichiometry of Tn:Tm:Ac7. The samples were diluted, immediately deposited on aminosilanized glass coverslips, and imaged in an epifluorescence microscope. From the co-localization of the three dye colors, we observe that (1) Tn-Tm binding to actin is a highly cooperative nearly all-or-nothing process, where actin had TnTm bound along its entire length or had no TnTm bound at all; (2) Tn-Tm not bound to actin are dissociated from each other. Filaments had different lengths, and Tn-Tm bound preferentially longer filaments. Regulated filaments were on average 4.75 times longer than unregulated filaments in presence and absence of Ca^{2+}. In the Mg^{2+}- and Ca^{2+}-saturated state, the average binding constant of TnTm to actin is 0.55 ± 0.08 uM-1 and 0.7 ± 0.2 uM-1 respectively. Dissociation rate of TnTm bound to f-actin at 20 was very slow (< 1 hr-1). Our results suggest that surface immobilized regulated actin, combined with single particle analysis and particle sorting, is a promising method for examining the structure of Tn as a member of regulated actin.

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The Accuracy of Cardiac Myofilament Simulations is Enhanced by Permitting Calcium-Independent Tropomyosin Transitions

Yasser Aboelkassem, Kimberly McCabe, **Stuart G. Campbell**.
Biomedical Engineering, Yale University, New Haven, CT, USA.

Conceptual and computational models have generally assumed that each regulatory unit (RU) of the thin filament remains in the blocked state until Ca^{2+} binds to troponin C. This includes our previous model (Campbell et al., *Biophys J* 98:2254, 2010), which simultaneously recapitulated key attributes of myofilament activation. However, the model failed to fully reproduce exchange experiments in which some fraction of myofilament troponin C is replaced with a non-Ca^{2+} binding mutant (xTnC). In simulations, xTnC caused much greater reductions in tension than were shown experimentally (Gillis et al., *J Physiol* 580:561, 2007). This effect was caused by the assumption of strong cooperative inhibition among nearest-neighbor RUs, which was required to produce basic myofilament activation behavior. We hypothesized that permitting some Ca^{2+}-independent RU activation while maintaining cooperative inhibition would reconcile this discrepancy. In a new model, blocked-to-closed RU transitions were allowed without bound Ca^{2+} but at greater energetic cost (ΔG). Monte Carlo simulations showed that reducing ΔG to a finite value of 4.7 kJ/mol maintained cooperative myofilament activation while reproducing xTnC experiments (Figure). These results suggest that thin filament function does not require perfect Ca^{2+} switch fidelity.